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(71) Applicant: **PHILLIPS PETROLEUM COMPANY**
5th and Keeler
Bartlesville Oklahoma 74004(US)

(72) Inventor: **Prevatt, William Dudley**
4524 Barlow Drive
Bartlesville, OK 74006(US)
Inventor: **Sreekrishna, Kotikanyad**
1060 Rolling Meadow Court
Bartlesville, OK 74006(US)

(74) Representative: **Dost, Wolfgang, Dr.rer.nat.,Dipl.-Chem. et al**
Patent- & Rechtsanwälte Bardehle . Pagenberg . Dost . Altenburg . Frohwitter . Geissler & Partner Galileiplatz 1 Postfach 86 06 20
W-8000 München 86(DE)

(54) **Expression of human serum albumin in pichia pastoris.**

(57) A process for the production of HSA in *Pichia pastoris* cells comprising cultivating *Pichia pastoris* cells capable of expressing HSA at a pH of about 5.7 to about 6.4 contemporaneously with the expression of HSA.

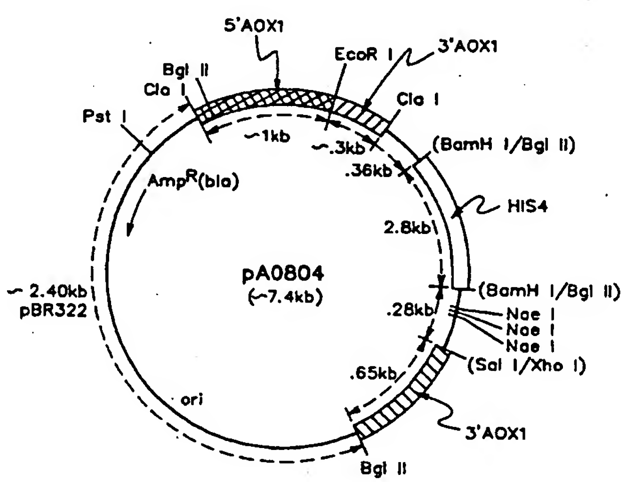

FIG. 1

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Field of the Invention

This invention relates to the field of recombinant DNA biotechnology. In one aspect, this invention relates to a process for the improved expression of secreted human serium albumin (HSA) in *Pichia pastoris*.

Background

Human serum albumin is the most abundant plasma protein of adults. The concentration of albumin is 40 mg/ml, or 160g of albumin circulating throughout the human body for a 70 Kg adult male. This protein maintains osmotic pressure and functions in the binding and transport of copper, nickel, calcium (weakly, at 2-3 binding sites), bilirubin and protoporphyrin, long-chain fatty acids, prostaglandins, steroid hormones (weak binding with these hormones promotes their transfer across the membranes), thyroxine, triiodothyronine, crystine, and glutathione. According to Peters, T. and Reed, R. G. in Albumin: Structure, Biosynthesis and Function, (Peter, T. and Sjoholm, J. eds.) 1977 p.11-20, over 10,000 kilograms of purified albumin are administered annually in the United States alone to patients with circulatory failure or with albumin depletion.

Currently the only commercial source of HSA is from fractionated blood. Considering the possible dangers of blood borne contaminants and pathogens, it would be a considerable contribution to the commercial production of HSA to develop alternate methods of producing HSA. With the advent of recombinant DNA technology, it is now possible to produce HSA by alternate methods.

HSA has also been expressed in *Saccharomyces cerevisiae* as disclosed by Etcheverry et al. in Bio/technology, August 1986, p. 726 and Arjum Singh in EPA 123,544. Etcheverry disclosed HSA expression intracellularly in a concentration of approximately 6 mg/l and the secretion of HSA which remained cell associated. Arjum Singh also disclosed the expression of HSA in *Saccharomyces cerevisiae* in combination with the α-factor promoter and signal sequence. Singh appears to have been able to achieve an intracellular production level of approximately 25 mg/l and a secreted production level of 3 mg/l. *Pichia pastoris* has also been used to express HSA as is disclosed in EPA 344,459. The concentration of HSA produced in *Pichia pastoris* appears to be about 89 ng HSA/mg of protein. Although the process for producing HSA in recombinant expression systems has been established by these experiments it would be desirable to optimize these processes to achieve the maximum possible HSA production.

Therefore, it would be a signficant contribution to the art to provide a process for increasing the yeild of HSA from the recombinant expression of HSA in microorganism such as *Pichia pastoris*.

Therefore, it is an object of this invention to provide a process for increasing the yield of HSA produced in a recombinant expression sytems.

Summary of the Invention

In accordance, we have discovered a process for improving the secreted expression of HSA in *Pichia pastoris* cells comprising:

(a) cultivating in a fermentation broth transformed *Pichia pastoris* cells capable of expressing HSA under conditions suitable for the sustained viability of said *Pichia pastoris* cells under suitable conditions for the expression of HSA by said *Pichia pastoris* cells; and maintaining the pH of said fermentation broth from a pH of from about 5.7 to about 6.0 contemporaneously with the expression of HSA.

Detailed Description of the Figures

Figure 1 provides a representation of plasmid pAO804 which contains a linear site-specific integrative vector in the fragment clockwise from BglII to BglII. The structural gene may be inserted in the unique EcoRI site of this plasmid. This plasmid may be recovered from the plasmid DNA of NRRL B-18114 by EcoRI digest and gel electrophoresis to recover a linear ~7.4 kb EcoRI fragment corresponding to Figure 1.

Figure 2 provides a representation of pHSA13 in circular form.

Figure 3 provides a restriction map of the AOX1 5' regulatory region isolated from *Pichia pastoris*.

Figure 4 provides a restriction map of the DAS1 5' regulatory region isolated from *Pichia pastoris*.

Figure 5 provides a restriction map of the AOX1 3' termination sequence isolated from *Pichia pastoris*.

Figure 6 provides a restriction map of the DAS1 3' termination sequence isolated from *Pichia pastoris*.

Figure 7 provides a representation of pHSA113 in linear form.

Figure 8 provides a representation of plasmid pAO807N which contains a linear site-specific integrative

vector in the fragment clockwise from NotI to NotI. The structural gene may be inserted in the unique EcoRI site of this plasmid.

Detailed Description

Generally *Pichia pastoris* is optimally grown at from about pH 4.8 to about pH 5.2. Between this pH range *Pichia pastoris* provided with a suitable nutrient media exhibits robust growth. This pH range also appears to result in high levels of expression of several foreign proteins such as hepatitis B surface antigen. This pH range also appeared to provide high levels of expression with human serum albumin (HSA). For example growing *Pichia pastoris* cells which had been transformed with a vector containing a HSA structural gene operably linked to a 5' regulatory region (i.e. a promoter) and a 3' termination sequence, the expression levels of HSA which had been obtained were approximately .71 to .81 grams/liters of HSA in the fermentation broth. However, we have been able to further increase this yield by at least 50% by taking the unprecedented step of shifting the pH of the fermentation broth from about 5.2 to in the range of from about pH 5.7 to about pH 6.4, with a preferred pH range of from about pH 5.7 to about pH 6.0 and most preferably a pH in the range of from pH 5.75 to pH 5.85. The increased secretion levels obtained in the upper limits of the pH range (i.e. from in the range of pH 6.0 to pH 6.4) have been confirmed in shake tube optimization studies which indicate that the presence of yeast extract and peptone together with aeration will provide optimal HSA secretion in shake tubes. However, the use of yeast extract, peptone and excess aeration is not believed necessary in large scale fermentation where the pH can be continuously monitored. We believe that this higher pH level will increase the yield of any *Pichia pastoris* strain transformed with an expression cassette containing a promoter and a structural gene encoding a signal sequence and the mature HSA protein. Further it would appear that this result will be applicable to a variety of heterologous structural genes which encode a signal sequence and a mature heterologous protein. Suitable heterologous proteins which may be expressed at higher levels utilizing this method include but are not limited to heterologous proteins selected from the group consisting of tissue plasminogen activator, albumins (such as human serum albumin), lysozymes (such as bovine lysozyme), interferons (such as gamma-interferon and beta-interferon) and invertase. Each of the heterologous structural genes utilized in the present invention must have a signal sequence operably linked to the 5' end of sequence coding for the mature heterologous protein to effect the secretion of the mature protein. For example the tissue plasmigen activator, human serum albumins, bovine lysozyme, beta-interferon, gamma-interferon and invertase proteins may all be secreted utilizing the native signal sequence. Furthermore these proteins may also be secreted utilizing secretion signal sequences from *Pichia pastoris* such as the acid phosphatase signal sequence disclosed in U.S. Patent Application Serial Number 07/627,539 filed December 14, 1990 by Richard Buckholz assigned to Phillips Petroleum Company (incorporated herein by reference) or the alpha-mating factor signal sequence from *Saccharomyces cerevisiae*.

Utilizing the present invention, HSA secretion levels of approximately 1-3 grams of authentic HSA per liter of fermentation broth have been obtained. This invention thus provides a means for the high level secretion of HSA. Achieving these levels of HSA production is a significant advancement over the prior production levels, since at the level of 1-3 grams per liter the recovery of HSA in high yields with high purities is possible.

To express the HSA structural gene, the gene must be operably linked to a 5' regulatory region and a 3' termination sequence, which forms an expression cassette which will be inserted into a host (usually a microorganism) via a vector (such as a plasmid or linear site-specific integrative vector). Operably linked as used in this context refers to a juxtaposition wherein the 5'regulatory region, structural gene, and 3' termination sequence are linked and configured so as to perform their normal function. 5' regulatory region or promoter as used herein means DNA sequences which respond to various stimuli and provide enhanced rates of mRNA transcription. 3' termination sequence are sequences 3' to the stop codon of a structural gene which function to stabilize the mRNA transcription product of the gene to which the sequence is operably linked (such as sequences which elicit polyadenylation). For the practice of this invention, it is preferred that the ATG of the structural gene be linked with as few intervening deoxyribonucleotides as possible to the 3' end of the 5' regulatory region, preferrably about 11 or less deoxyribonucleotides and most preferably 8 or less deoxyribonucleotides. It is also preferred that the adenine and thymine content of the intervening deoxyribonucleotides be in the range of from about 55 percent to about 64 percent. Further, it appears that there are nucleotide preferences for certain specific locations. Counting left from the ATG codon of the structural gene with the first position left being the -1 position, it appears that adenine or cytosine is the most preferred deoxyribonucleotide, in the -2 position the most preferred deoxyribonucleotide is either adenine or thymine, in the -3 position the most preferred deoxyribonucleotide is

adenine or thymine and the most preferred nucleotide at the -4 position is adenine, thymine or cytosine. Currently, it is preferred that the AOX1 or DAS1 5' regulatory region having the restriction maps of Figures 3 and 4 or, the sequences provided as SEQ ID No: 1 and SEQ ID No: 2, respectively, be linked at their 3' end of the sequence to the ATG start codon of the HSA structural gene. One example of an appropriate linkages for the AOX1 5' regulatory region is illustrated below:

Table I

Construct Designation	End of the 5' Regulatory Region for AOX 1	Deoxyribonucleotide intervening before ATG start condon
pHSA413	5' - TTCGAAACG	5' - NONE

Several 5' regulatory regions have been characterized and can be employed in conjunction with the expression of HSA in *Pichia pastoris*. Exemplary 5' regulatory regions are the primary alcohol oxidase (AOX1), dihydroxyacetone synthase (DAS1), glyceraldehyde-3-phosphate dehydrogenase gene (GAP), acid phosphatase gene (PHO1) and the p40 regulatory regions, derived from *Pichia pastoris* and the like. The AOX1 5' regulatory region, DAS1 5' regulatory region and p40 5' regulatory region are described in U.S. Patent 4,855,231, incorporated herein by reference. The GAP 5' regulatory region is disclosed in EPA 374,913 published June 27, 1990, incorporated herein by reference. The PHO1 5' regulatory region is disclosed in U.S. Patent Application 07/672,539 filed December 14, 1990, assigned to Phillips Petroleum Company. The presently preferred 5'regulatory regions employed in the practice of this invention are those characterized by their ability to respond to methanol-containing media, such regulatory regions selected from the group consisting of AOX1, and DAS1. The most preferred 5' regulatory region for the practice of this invention is the AOX1 5' regulatory region.

3' termination sequences should be utilized in the expression cassette as discussed above. 3' termination sequences may function to terminate, polyadenylate and/or stabilize the messenger RNA coded for by the structural gene when operably linked to a gene, but the particular 3' termination sequence is not believed to be critical to the practice of the present invention. A few examples of illustrative sources for 3' termination sequences for the practice of this invention include but are not limited to the *Hansenula polymorpha* and *Pichia pastoris* 3' termination sequences. Preferred are those derived from *Pichia pastoris* such as those selected from the group consisting of the 3' termination sequences of AOX1 gene, DAS1 gene, p40 gene GAP gene, PHO1 gene and HIS4 gene. Particularly preferred is the 3' termination sequence of the AOX1 gene.

Pichia pastoris may be transformed with a variety of HSA structural genes (in the inventive transformants discussed herein the HSA structural gene encodes both a signal sequence and a mature HSA protein). HSA structural genes have been sequenced by Lawn et al. Nuc. Acids Res. 9:6105 (1981), and Dugaiczyk et al., Proc. Natl. Acad. Sci. USA 79:71 (1982). These genes may also be obtained by reisolation of the genes by the technique of Lawn et al., Dugaiczyk et al. or synthesized in vitro by a custom gene manufacturer such as British Biotechnology, Ltd. One possible method of obtaining a HSA gene would be to screen a human liver cDNA library with oligonucleotide probes or screen a human liver cDNA expression library with anti-HSA antisera to identify HSA expressing cDNAs. One suitable HSA structural gene is provided in SEQ ID NO: 3. Once a structural gene for HSA is recovered, it may be necessary to further tailor the gene. Following the isolation of an HSA structural gene, the gene is inserted into a suitable *Pichia pastoris* vector such as a plasmid or linear site-specific integrative vector.

Plasmid-type vectors have long been one of the basic elements employed in recombinant DNA technology. Plasmids are circular extra-chromosomal double-stranded DNA found in microorganisms. Plasmids have been found to occur in single or multiple copies per cell. Included in plasmid DNA is the information required for plasmid reproduction, e.g. an autonomous replication sequence such as those disclosed by James M. Cregg in U.S. Patent 4,837,148, issued June 6, 1989, incorporated herein by reference. Additionally one or more means of phenotypically selecting the plasmid in transformed cells may also be included in the information encoded in the plasmid.

Suitable integrative vectors for the practice of the present invention are the linear site-specific integrative vectors described by James M. Cregg, in U.S. Patent 4,882,279, issued November 21, 1989, which is incorporated herein by reference. These vectors comprise a serially arranged sequence of at least 1) a first insertable DNA fragment; 2) a selectable marker gene; and 3) a second insertable DNA fragment. An expression cassette containing a heterologous structural gene is inserted in this vector between the first and second insertable DNA fragments either before or after the marker gene. Alternatively, an expression

cassette can be formed *in situ* if a regulatory region or promoter is contained within one of the insertable fragments to which the structural gene may be operably linked.

The first and second insertable DNA fragments are each at least about 200 nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be transformed. The various components of the integrative vector are serially arranged forming a linear fragment of DNA such that the expression cassette and the selectable marker gene are positioned between the 3' end of the first insertable DNA fragment and the 5' end of the second insertable DNA fragment. The first and second insertable DNA fragments are oriented with respect to one another in the serially arranged linear fragment as they are oriented in the parent genome.

Nucleotide sequences useful as the first and second insertable DNA fragments are nucleotide sequences which are homologous with separate portions of the native genomic site at which genomic modification is to occur. For example, if genomic modification is to occur at the locus of the alcohol oxidase gene, the first and second insertable DNA fragments employed would be homologous to separate portions of the alcohol oxidase gene locus. Examples of nucleotide sequences which could be used as first and second insertable DNA fragments are deoxyribonucleotide sequences selected from the group consisting of the *Pichia pastoris* alcohol oxidase (AOX1) gene, dihydroxyacetone synthase (DAS1) gene, p40 gene glyceraldehyde-3-phosphate dehydrogenase (GAP), acid phosphatase (PHO1) and HIS4 gene. The AOX1 gene, DAS1 gene, p40 gene and HIS4 genes are disclosed in U.S. Patents 4,855,231 and 4,885,242 both incorporated herein by reference. The designation DAS1 is equivalent to the DAS designation originally used in U.S. Patents 4,855,231 and 4,885,242. The GAP gene is disclosed in EPA 374,913 published June 27, 1990 incorporated herein by reference. The PHO1 gene is disclosed in U.S. Patent Application 07/672,539 filed December 14, 1990, assigned to Phillips Petroleum Company, incorporated herein by reference.

The first insertable DNA fragment may contain an operable regulatory region which may comprise the regulatory region utilized in the expression cassette. The use of the first insertable DNA fragment as the regulatory region for an expression cassette is a preferred embodiment of this invention. Figure 1 provides a diagram of a vector utilizing the first insertable DNA fragment as a regulatory region for a cassette. Optionally, as shown in Figure 1, an insertion site or sites and a 3' termination sequence may be placed immediately 3' to the first insertable DNA fragment. This conformation of the linear site-specific integrative vector has the additional advantage of providing a ready site for insertion of a structural gene without necessitating the separate addition of a compatible 3' termination sequence.

If the first insertable DNA fragment does not contain a regulatory region, a suitable regulatory region will need to be inserted linked to the structural gene, in order to provide an operable expression cassette. Similarly, if no 3' termination sequence is provided at the insertion site to complete the expression cassette, a 3' termination sequence can be operably linked to the 3' end of the structural gene.

It is also highly desirable to include at least one selectable marker gene in the DNA used to transform the host strain. This facilitates selection and isolation of those organisms which have incorporated the transforming DNA. The marker gene confers a phenotypic trait to the transformed organism which the host did not have, e.g. restoration of the ability to produce a specific amino acid where the untransformed host strain has a defect in the specific amino acid biosynthetic pathway, or provides resistance to antibiotics and the like. Exemplary selectable marker genes may be selected from the group consisting of the HIS4 gene (disclosed in U.S. Patent 4,885,242) and the ARG4 gene (disclosed in U.S. Patent 4,818,700 incorporated herein by reference) from *Pichia pastoris* and *Saccharomyces cerevisiae*, the invertase gene (SUC2) (disclosed in U.S. Patent 4,857,467 incorporated herein by reference) from *Saccharomyces cerevisiae*, or the G418^R/kanamycin resistance gene from the *E. coli* transposable elements Tn601 or Tn903.

Those skilled in the art recognize that additional DNA sequences can also be incorporated into the vectors employed in the practice of the present invention, such as, for example, bacterial plasmid DNA, bacteriophage DNA, and the like. Such sequences enable the amplification and maintenance of these vectors in bacterial hosts.

The insertion of the HSA structural gene into suitable vectors may be accomplished by any suitable technique which cleaves the chosen vector at an appropriate site or sites and results in at least one operable expression cassette containing the HSA structural gene being present in the vector. Ligation of the HSA structural gene may be accomplished by any appropriate ligation technique such as utilizing T4 DNA ligase.

The initial selection, propagation, and optional amplification of the ligation mixture of the HSA structural gene and a vector is preferably performed by transforming the mixture into a bacterial host such as *E. coli* (although the ligation mixture could be transformed directly into a yeast host but, the transformation rate would be extremely low). Suitable transformation techniques for *E. coli* are well known in the art.

Additionally, selection markers and bacterial origins of replication necessary for the maintenance of a vector in a bacterial host are also well known in the art. The isolation and/or purification of the desired plasmid containing the HSA structural gene in an expression system may be accomplished by any suitable means for the separation of plasmid DNA from the host DNA. Similarly the vectors formed by ligation may be tested, preferably after propagation, to verify the presence of the HSA gene and its operable linkage to a regulatory region and a 3' termination sequence. This may be accomplished by a variety of techniques including but not limited to endonuclease digestion, gel electrophoresis, or Southern hybridization.

Transformation of plasmids or linear vectors into yeast hosts may be accomplished by suitable transformation techniques including but not limited to those taught by Cregg and Barringer, U.S. Patent 4,929,555; Hinnen et al., Proc. Natl. Acad. Sci. 75, (1978) 1929; Ito et al., J. Bacteriol. 153, (1983) 163; Cregg et al. Mol. Cell Biol. 5 (1985), pg. 3376; D. W. Stroman et al., U.S. Patent 4,879,231, issued November 7, 1989; or Sreekrishna et al., Gene, 59 (1987), pg. 115. Preferable for the practice of this invention is the transformation technique of Cregg et al. (1985). It is desirable for the practice of this invention to utilize an excess of linear vectors and select for multiple insertions by Southern hybridization.

The yeast host for transformation may be any suitable methylotrophic yeast. Suitable methylotrophic yeasts include but are not limited to yeast capable of growth on methanol selected from the group consisting of the genera *Hansenula and Pichia.* A list of specific species which are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982). Presently preferred are methylotrophic yeasts of the genus *Pichia* such as the auxotrophic *Pichia pastoris* GS115 (NRRL Y-15851); *Pichia pastoris* GS190 (NRRL Y-18014) disclosed in U.S. Patent 4,818,700; and *Pichia pastoris* PPF1 (NRRL Y-18017) disclosed in U.S. Patent 4,812,405. Auxotrophic *Pichia pastoris* strains are also advantageous to the practice of this invention for their ease of selection. It is recognized that wild type *Pichia pastoris* strains (such as NRRL Y-11430 and NRRL Y-11431) may be employed with equal success if a suitable transforming marker gene is selected, such as the use of SUC2 to transform *Pichia pastoris* to a strain capable of growth on sucrose or an antibiotic resistance marker is employed, such as G418.

Transformed *Pichia pastoris* cells can be selected for by using appropriate techniques including but not limited to culturing previously auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype ("methanol slow"), or culturing in the presence of an antibiotic which is toxic to the yeast in the absence of a resistance gene contained in the transformant.

Isolated transformed *Pichia pastoris* cells are cultured by appropriate fermentation techniques such as shake flask fermentation, high density fermentation or the technique disclosed by Cregg et al. in, High-Level Expression and Efficient Assembly of Hepatitis B Surface Antigen in the Methylotrophic Yeast, *Pichia Pastoris* 5 Bio/Technology 479 (1987). Isolates may be screened by assaying for HSA production to identify those isolates with the highest HSA production level.

The cultivation of transformed *Pichia pastoris* can be conducted in an aqueous continuous or batch-fed manner, utilizing a variety of carbon-energy sources and/or nutrient sources. For the practice of the present invention, batch-fed fermentation is preferred. Suitable carbon-energy sources for growing *Pichia pastoris* include but are not limited to the carbon-energy source selected from the group consisting of methanol, glycerol, sorbitol, glucose, fructose and combinations of any two or more thereof. Preferred carbon-energy sources for growing *Pichia pastoris* are carbon-energy sources selected from the group consisting of methanol, glycerol, and combinations thereof. A suitable nutrient source or media for *Pichia pastoris* would include at least one nitrogen source, at least one phosphate source, at least one source of minerals such as iron, copper, zinc, magnesium, manganese, calcium, and other trace elements, and vitamins (such as biotin, pantothenic acid, and thiamine as required).

Suitable sources of at least one carbon-energy source and nutrients can be obtained from a variety of sources or may consist of a single source. However, preferred are at least one carbon-energy source and/or nutrient sources which have a defined character. One carbon-energy source and/or nutrient composition which has proven effective is:

Table II

Carbon-Energy Source and Nutrients	
Component per Liter of Water	
Carbon-energy Source (glycerol)	50.0 g/l
H_3PO_4 (85%)	21 ml/l
$CaSO_4 \cdot 2H_2O$	0.9 g/l
K_2SO_4	14.28 g/l
$MgSO_4 \cdot 7H_2O$	11.7 g/l
KOH	3.9 g/l
Peptone	10.0 g/l
[1]Yeast Extracts	5.0 g/l
[2]Minerals and Trace Metals	1.0 ml/l

[1]Yeast extract is Amberex™ 1003 which is available from and a trademark of Universal Foods Corporation, Milwaukee, Wisconsin.
[2]Minerals and trace metals are $FeSO_4 \cdot 7H_2O$ 65.0 g/l, $CuSO_4 \cdot 5H_2O$ 6.0 g/l, $ZnSO_4 \cdot 7H_2O$ 20 g/l, $MnSO_4$ 3.0 g/l and H_2SO_4 5.0 ml/l

The yeast extracts utilized in the present invention include but are not limited to yeast extracts selected from the group consisting of Amberex™ 1003 and Bacto™ Yeast Extract (Difco Laboratories Incorporated). Alternatively, corn steep liquor could be used to replace yeast extracts as a source of nitrogen.

Trace metals utilized in the present invention are those trace metals generally utilized in the yeast growth provided in an amount sufficient to not limit the growth rate or HSA production of *Pichia pastoris* which include but are not limited to trace metals selected from the group consisting of cobalt, molybdenum, iron, copper, zinc, and manganese.

The fermentation temperature should generally range from about 20°C to about 35°C and preferably should be about 30°C.

The dissolved oxygen content in the fermentation vessel where the fermentation is conducted in a batch-fed manner may range from about 20 percent to about 80 percent of saturation and preferably will range from about 30 percent to about 60 percent of saturation.

After the *Pichia pastoris* strains transformed with a vector containing the HSA structural gene have been cultivated to a high density, the transformed strains should then be induced to express HSA at a pH of from about 5.7 to about 6.0. For example, if this technique is employed with a strain transformed with a linear expression cassette containing a methanol inducible regulatory region, the culture would first be grown to the desired density on minimal salts, biotin and 5 percent glycerol by weight. The pH should be adjusted to 5.8 (with ammonia) with a temperature of about 30°C and a dissolved oxygen concentration of about 20 percent of saturation. After the glycerol is exhausted, the promoter would be induced by beginning a slow methanol feed. The fed should provide methanol to the culture at a rate at least sufficient to maintain the viability of the culture but the maximum methanol concentration in contact with the culture should be no more than about 5.0 percent by weight. The HSA secretion can be monitored during the methanol feeding by sampling the HSA present in the cell free broth. Suitable test for quantifying the amount of HSA produced are known to those skilled in the art, such as running polyacrylamide gels. The methanol feed should be continued until the HSA concentration reaches an acceptable level. Generally, the HSA production will peak after about 120 hours on methanol feed.

If the transformed *Pichia pastoris* cells are grown in shake tubes or shake flasks instead of pH controlled fermenter, additional steps should be taken to assure the maximum yields of secreted proteins, such as HSA. Specifically, it is recommended that the media used be modified from that used in fermenter to a complex media and the aeration be increased. The complex media utilized in the shake flasks and shake tubes should contain added amino acids. The amino acids may be in a defined media containing glutamic acid, methionine, lysine, leucine, isoluecine and other amino acids or through a complex media supplement, such as yeast extract or casamino acids. The relative concentrations of the added amino acids should generally range from about 2.5 mg/liter to about 10 mg/liter with the preferred range being from about 4 mg/liter to about 6 mg/liter of glutamic acid, methionine, lysine, leucine and isoluecine and from about 0.5 mg/liter to about 3 mg liters of the remaining amino acid (however,histidine may be omitted entirely from the added amino acids). If yeast extract is used in place of the added amino acids, it is preferred that the yeast extract be provided in a concentration of in the range of from about 1 g/liter to

about 15 g/liter be utilized in the media and most preferably the yeast extract will be provided in a concentration of 10 g/liter. It has also been found desirable to add peptone to the media to improve secretion in shake tubes and shake flasks. For optimum secretion that peptone be used with the yeast extract in a concentration of from in the range of from about 1 g/liter to about 50 g/liter, and most preferably in a concentration of about 20 g/liter. As a guideline, it is generally recommended that the peptone concentration be twice the yeast extract concentration.

Aeration in shake flask and shake tube growth of transformed *Pichia pastoris* appears to be an important parameter in obtaining optimum secretion. To insure adequate aeration, it is recommended that shake tube or flask have a large aperture covered with an air permeable cap. Suitable air permeable caps can be made of a loose filter material, such as cheese cloth. One suitable shake flask for this invention is the Tunair shake flask. Generally, low baffle shake flasks are also recommended to avoid excessive foaming. Shaker speed for aeration is recommended to be in the range of from about 250 rpms to about 300 rpms.

After a suitable cell density is achieved in the shake flask or shake tube, the cells may be recovered then resuspended in a medium containing methanol in place of the carbon source used for growth to induce the secretion of protein. The flask or shake tubes may then be monitored on a regular basis to determine when the desired level of production has been achieved.

The invention will now be described in greater detail in the following non-limiting examples.

Examples

General information pertinent to the Examples:

Strains

Pichia pastoris GS115 (his 4) NRRL Y-15851
E. coli DG75' (hsd1, leu6, lacY, thr-1, supE44, tonA21, lambda$^-$)

Buffers, Solutions and Media

The buffers, solutions, and media employed in the following examples have the compositions given below:

dH_2O	deionized H_2O that has been treated with a milli-Q (Millipore) reagent water system.
1M Tris buffer	121.1 g Tris base in 800 mL of H_2O; adjust pH to the desired value by adding concentrated (35%) aqueous HCl; allow solution to cool to room temperature before final pH adjustment, dilute to a final volume of 1 L.
TE buffer	1.0 mM EDTA in 0.01 M (pH 8.0) Tris bufffer
SED	1 M sorbitol 25 mM EDTA 50 mM DTT, added prior to use --adjust to pH 8
SCE	9.1 g sorbitol 1.47 g Sodium citrate 0.168 g EDTA --pH to 5.8 with HCl in 50 ml dH_2O and autoclave
CaS	1 M sorbitol 10 mM $CaCl_2$ --filter sterilize
SOS:	1 M sorbitol 0.3x YPD 10 mM $CaCl_2$
PEG	20% polyethylene glycol-3350 10 mM $CaCl_2$

	10 mM Tris-HCl (pH 7.4)
	--filter sterilize
Solution A	0.2 M Tris-HCl (pH 7.5)
	0.1 M $MgCl_2$
	0.5 M NaCl
	0.01 M dithiothreitol (DTT)
Solution B	0.2 M Tris-HCl (pH 7.5)
	0.1 M $MgCl_2$
	0.1 M DTT
Solution C (keep on ice)	4 µl solution B
	4 µl 10 mM dATP
	4 µl 10 mM dTTP
	4 µl 10 mM dGTP
	4 µl 10 mM dCTP
	4 µl 10 mM ATP
	5 µl T_4 ligase (2 U/µl)
	12 µl H_2O
	Recipe for Solution C was modified from Zoller & Smith
LB Broth, 1 liter	5.0 g yeast extract
	10.0 g tryptone
	5.0 g NaCl
10X Transfer Buffer	96.8 g Trizma Base
	9.74 g glycine
	water to 1 liter
Ligation Buffer	50 mM Tris-HCl (pH 7.4)
	10 mM $MgCl_2$
	10 mM dithiothreitol
	1 mM ATP
Phosphatase Buffer	50 mM Tris-HCl (pH 9.0)
	1 mM $MgCl_2$
	1 mM $ZnCl_2$
	1 mM spermidine
Bsu36I buffer	100 mM NaCl
	10 mM Tris-HCl (pH 7.4)
	10 mM $MgCl_2$
	100 µg/ml BSA
Csp45I buffer	60 mM NaCl
	10 mM Tris-HCl, pH 7.5
	7 mM $MgCl_2$
	100 µg/ml BSA
REact 1 buffer	50 mM Tris-HCl, pH 8.0
	10 mM $MgCl_2$
	100 µg/ml BSA
REact 2 buffer	REact 1 buffer + 50 mM NaCl
REact 3 buffer	REact 1 buffer + 100 mM NaCl
HS buffer	50 mM Tris-HCl, pH 7.5
	10 mM $MgCl_2$
	100 mM NaCl
	1 mM DTT
	100 µg/ml BSA
10X Basal Salts	42 mls Phosphoric Acid, 85%
	1.8 g Calcium Sulfate • $2H_2O$
	28.6 g Potassium Sulfate
	23.4 g Magnesium Sulfate • $7H_2O$
	6.5 g Potassium Hydroxide
Ptm_1 Trace Salts Solution	6.0 g Cupric Sulfate • $5H_2O$
	0.08 g Sodium Iodide

	3.0 g Manganese Sulfate • H_2O
	0.2 g Sodium Molybdate • H_2O
	0.02 g Boric Acid
	0.5 g Cobalt Chloride
	20.0 g Zinc Chloride
	65.0 g Ferrous Sulfate • H_2O
	0.20 g Biotin
	5.0 mls Sulfuric Acid
YPD (yeast extract peptone dextrose medium)	10 g bacto yeast extract 20 g peptone 10 g dextrose water to 1 liter
MGY (minimal glycerol medium)	13.4 g yeast nitrogen base with ammonium sulfate, and without amino acids 400 µg biotin 10 ml glycerol water to 1 liter
MM (minimal methanol medium)	Same as MGY, except that 5 ml methanol is used in the place of 10 ml glycerol.
SDR (supplemented dextrose regeneration medium):	13.4 g yeast nitrogen base with ammonium sulfate and without amino acids 400 µg biotin 182 g sorbitol 10 g glucose 2 g Histidine assay mix (Gibco) 50 mg glutamine 50 mg methionine 50 mg lysine 50 mg leucine 50 mg isoleucine 10 g agarose water to 1 liter
BMGR (Buffered minimal glycerol-enriched medium)	100 ml/liter Potassium phosphate buffer, (pH 6.0) 13.4 grams/liter Yeast nitrogen base with ammonium sulfate 400 µg/liter biotin 10 ml/liter glycerol Amino acids glutamic acid, methionine, lysine, leucine and isoleucine: each at 5 mg/liter; all the other amino acids except histidine at 1 mg/liter Nucleotides adenine sulfate, guanine hydrochloride, uracil, and xanthine, each at 40 µg/liter Vitamins thiamine hydrochloride, riboflavin, and calcium pantothenate, each at 2 µg/liter; pyridoxide hydrochloride and nicotinic acid, each at 4 µg/liter; pyridoxamine hydrochloride and pyridoxal hydrochloride, each at 1 µg/liter; para-amino benzoic acid at 0.3 µg/liter; folic acid at 0.03 µg/liter Trace minerals magnesium sulfate at 800 µg/liter; ferrous sulfate at 40 µg/liter;

	manganese sulfate at 80 µg/liter; sodium chloride at 40 µg/liter
BMGY (Buffered minimal glycerol-complex medium)	100 ml/liter potassium phosphate buffer, (pH 6.0) 13.4 grams/liter yeast nitrogen base with ammonium sulfate and without amino acids biotin at 400 µg/liter glycerol at 10 ml/liter yeast extract at 10 g/liter peptone at 20 g/liter
BMMR (Buffered minimal methanol-enriched medium)	Same as BMGR, with the exception that 5 ml methanol/liter is added in the place of glycerol
BMMY (Buffered minimal methanol -complex medium)	Same as BMGY, with the exception that 5 ml methanol/liter is added in the place of glycerol

Techniques

Suitable techniques for recombinant DNA lab work may be found in many different references including but not limited to: Methods in Enzymology, (Orlando, FL: Academic Press, Inc.), particularly Volume 152, published as, Guide to Molecular Cloning Techniques, by Berger and Kimmel (Orlando, FL: Academic Press, Inc., 1987) and Molecular Cloning/A Laboratory Manual, by Sambrook et al., 2d ed. (Cold Spring Harbor Laboratory Press, 1989) and which are all hereby incorporated by reference.

Example I

Construction of 5'-exact HSA expression vector pHSA313

The pHSA313 vector was constructed to provide a vector with an exact linkage between the 3' end of the native AOX1 5' regulatory region (promoter) and the start codon of the HSA structural gene.

A. Creation of pHSA113ΔCla

About 200 ng of pHSA113, disclosed in European Patent Application 0 344 459 which is herein incorporated by reference, (see Figure 7) was digested at 37°C for 1 hour with 1 unit of ClaI in 20 µl of REact 1 buffer. The digestion mixture was brought to 100 µl with water and extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 V/V), followed by extracting the aqueous layer with an equal volume of chloroform:isoamyl alcohol (24:1). The DNA in the aqueous phase was precipitated by adjusting the NaCl concentration to 0.2 M and adding 3 volumes of cold ethanol. The mixture was allowed to stand on ice (4°C) for 10 minutes and the DNA precipitate was collected by centrifugation for 30 minutes at 10,000 x g in a microfuge at 4°C. The DNA pellet was washed 2 times with 70% aqueous cold ethanol. The washed pellet was vacuum dried and dissolved in 10 µl water to which 2 µl of 10 x ligation buffer, 2 µl of 1 mg/ml BSA, 6 µl of water and 1 unit T_4 DNA ligase were added. The mixture was incubated overnight at 4°C and a 10 µl aliquot was used to transform E. coli DG75' (Maniatis, et al.) to obtain pHSA113ΔCla, which represents the deletion of HIS4 and 3'AOX1, along with small stretches of pBR322 sequences used to link these sequences. The deletion of the HIS4, 3' AOX1 and pBR322 sequences removes one of two Csp45I sites present in the pHSA113 vector. The remaining Csp45I site is in the AOX1 5' regulatory region (promoter).

B. Creation of pXHSA113ΔCla

Digest 5 µg of pHSA113ΔCla for 1 hour at 37°C with 10 units of BstEII in 100 µl of REact 2 buffer. The digestion mixture was extracted with phenol and precipitated as detailed in step A. The DNA precipitate was dissolved in 100 µl of Csp45I buffer and digested at 37°C for 2 hours in the presence of 10 units of Csp45I. The digested DNA was then phenol extracted and precipitated as described in step A. The DNA precipitate was dissolved in 20 µl of water and 10 µl aliquots were loaded on 2 neighboring wells of a 0.9% agarose gel. Following electrophoresis, the gel portion corresponding to one of the lanes was stained and this was used to locate the position of the Csp45I-BstEII fragment of pHSA113ΔCla in the unstained lane. The gel portion containing the larger Csp45I-BstEII fragment was excised out and the DNA in the gel was

electroeluted into 500 µl of 5 mM EDTA, pH 8.0. The DNA solution was phenol extracted as detailed in step A and the DNA precipitate was dissolved in 100 µl water. The larger Csp45I-BstEII fragment was then ligated with the BstEII-Csp45I oligonucleotide linker described below. An aliquot (10 µl) was ligated overnight at 4°C with 20 ng of annealed linker oligonucleotides 5'-CGAAACG ATG AAG TGG (SEQ ID NO:4) and 5'-GTTACCCACTTCATCGTTT (SEQ ID NO:5) in 20 µl ligase buffer containing 100 µg/ml BSA and 1 unit of T_4 DNA ligase. The ligation mixture was used to transform E. coli DG75' to obtain pXHSA113ΔCla. The pXHSA113ΔCla vector by virtue of the linker described above has an exact linkage between the 3' end of the native AOX1 5' regulatory region (promoter) and the HSA ATG start codon with no extraneous DNA sequences.

C. Creation of pHSA313

1 µg of pXHSA113ΔCla was digested for 4 hours at 37°C with ClaI in 100 µl of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200 µl reaction volume for 30 minutes at 37°C. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in water at a concentration of approximately 10 ng/µl as described in step A and stored at -20°C.

1 µg of pAO807N (Figure 8, construction of which is described in European Patent Application 0 344 459) was digested for 4 hours at 37°C with PstI in 100 µl of REact 2 buffer. The digested DNA was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in a 200 µl reaction volume for 15 minutes at 55°C. At the end of 15 minutes another 10 units of phosphatase was added and incubated for 15 minutes. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated as described in step A. DNA was digested for 4 hours at 37°C with 5 units of ClaI in 100 µl REact 1 buffer containing 100 µg/ml BSA, followed by phenol extraction and precipitation of DNA as outlined in step A. The DNA precipitate was dissolved in water at a concentration of approximately 20 ng/µl.

Approximately 100 ng (10 µl) of ClaI cleaved-phosphatased pXHSA113ΔCla was mixed with approximately 80 ng of PstI digested-phosphatased and ClaI-cleaved pAO807N (4 µl), 4 µl of 5X ligase buffer, 2 µl of 1 mg/ml BSA and ligated overnight at 4°C using 1 unit of T_4 DNA ligase. The ligation mixture was used to transform E. coli DG75' to obtain pHSA313. The pHSA313 plasmid from this ligation contains the complete pXHSA113ΔCla sequence linked to the HIS4 gene and the AOX1 3' second insertable sequence derived from AO807N. The relative orientation of the components of the pHSA313 plasmid is the same as that shown in Figure 7 for plasmid pHSA113.

Example II

Construction of Expression Vector pPGP1

The expression vector pPGP1 was constructed in the following manner. pXHSA113ΔCla (see Example I) was digested with Bsu36I and PvuII (partial) and the vector backbone was isolated. An HSA structural gene on a PvuII-Bsu36I fragment analogous to the structural gene contained in pHSA113 (disclosed in European Patent Application 0 344 459) was ligated to this vector backbone to obtain pPGP1ΔCla. About 100 ng of pPGP1ΔCla was digested with ClaI at 37°C for 1 hour. The DNA was recovered as in Example I. About 100 ng of pAO807N (shown herein in Figure 8 and disclosed in European Patent Application 0 344 459) was digested with PstI, alkaline phosphatase treated and then digested with ClaI as detailed in Example I C. This fragment was then ligated to ClaI cleaned, alkaline phosphatase treated pPGP1ΔCla to obtain pPGPI. (GS115 pPGP1-9-6 is a clone which was obtained by transformation of *Pichia pastoris* GS115 with pPGP1 and this clone was used in fermentation).

Example III

Construction of 5' & 3' exact HSA expression plasmid pHSA413

The pHSA413 vector was constructed to provide a vector with an exact linkage between the 3' end of the AOX1 5' regulatory region and the start codon fo the HSA structural gene as well as an exact linkage between the 5' end of the AOX1 3' termination sequence and the 3' end of the HSA structural gene.

A.. Creation of pXXHSA113ΔCla

1 µg of pXHSA113ΔCla was digested for 4 hours at 37°C with 10 units of EcoRI in 100 µl REact 3 buffer. The digestion mixture was phenol extracted and DNA precipitated as detailed in Example VI. DNA precipitate was dissolved in 20 µl water and digested for 1 hour at 37°C with 20 units of Bsu36I in 100 µl of Bsu36I buffer. The digestion mixture was phenol extracted, DNA precipitated and dissolved in 100 µl of water as detailed in Example VI. Approximately 100 ng of EcoRI and Bsu36I-cleaved DNA was mixed with 10 ng of annealed oligonucleotides 5'-TTAGGCTTATAAG (SEQ ID NO:6) and 5'-AATTCTTATAAGCC (SEQ ID NO:7) and ligated overnight at 4°C in 20 µl of T_4 DNA ligase buffer containing 100 µg/ml BSA and 10 units of T_4 DNA ligase. The ligation mixture was used to transform E. coli to obtain pXXHSA113ΔCla. In this plasmid the sequence between Bsu36I and EcoRI (SEQ ID NO:8) present in pxHSA113ΔCla shown below

```
Bsu36I
5'CCTTAGGCTTATAACATCTCTACATTTAAAAGCATCTCAGCCTACCATGAGAATAAGAGAAAGAAAATGAAGATCA
AAAGCTTATTCATCTGTGTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCTAAAAAACATAAATTTCTTTAATC
ATTTTGCCTCTTTTTCTCTGTGCTTCAATTAATAAAAAATGGAAAGAATCTAAAAAAAAAAAAAAAAAAAGGAATTC
                                                                         EcoRI
```

```
is replaced by 5'CC TTA GGC TTA TAA GAATTC (SEQ ID NO:9)
                 Bsu36I         EcoRI
```

B. Creation of pHSA413

1 µg of pXXHSA113ΔCla was digested for 4 hours at 37°C with ClaI in 100 µl of REact 1 buffer. Following digestion the reaction mixture was adjusted to alkaline phosphatase buffer conditions and treated with 10 units of calf intestinal alkaline phosphatase in 200 µl reaction volume for 30 minutes at 37°C. Phosphatase treatment was terminated by phenol extraction and the DNA was precipitated and dissolved in water at a concentration of approximately 10 ng/µl as described in step A and stored at -20°C.

Approximately 100 ng (10 µl) of ClaI cleaved-phosphatased pXXHSA113ΔCla was mixed with approximately 80 ng (4 µl) of PstI digested phosphatased and ClaI-cleaved pAO807N (see paragraph 2 in step 3 of Example VI), 4 µl of 5X ligase buffer, 2 µl of 1 mg/ml BSA and ligated overnight at 4° C using 1 unit of T_4 DNA ligase. The ligation mixture was used to transform E. coli DG75' to obtain pHSA413. The pHSA413 plasmid from theis ligation contains the complete pXHSA113ΔCla sequence linked to the HIS4 gene and the AOX1 3' second insertable sequence derived from AO807N. The relative orientation of the components of the pHSA413 plasmid is the same as that shown in Figure 7 for plasmid pHSA113.

Example IV

Transformation of *Pichia pastoris* with pHSA313, pHSA413, and pPGP1

A.. Vector preparation

About 10 µg each of pHSA313, pHSA413, pPGP1, and pAO807N (negative control) were digested for 12 hours at 37°C in 200 µl of HS buffer with 50 units of NotI. The digested DNA samples were phenol extracted, precipitated as described in Example VI, dissolved in 20 µl of CaS, and were then used for transformation of *Pichia pastoris* GS115. About 10 µg each of pHSA313, pHSA413, and pAO807N were also digested with 20 units of SstI for 12 hours at 37°C in 200 µl of REact 2 buffer containing 100 µg/ml of BSA. The digested DNA samples were extracted with phenol, precipitated as described in Example VI and dissolved in 20 µl of CaS.

B. Cell Growth

Pichia pastoris GS115 (NRRL Y-15851) was inoculated into about 10 ml of YPD medium and shake cultured at 30°C for 12-20 hours. 100 ml of YPD medium was inoculated with a seed culture to give an OD_{600} of about 0.001. The medium was cultured in a shake flask at 30°C for about 12-20 hours. The

culture was harvested when the OD_{600} was about 0.2-0.3 by centrifugation at 1555 g for 5 minutes using a Sorvall RB5C.

C. Preparation of Spheroplasts

The cells were washed in 10 ml of sterile water, and then centrifuged at 1500 g for 5 minutes. (Centrifugation is performed after each cell wash at 1500 g for 5 minutes using a Sorvall RT6000B unless otherwise indicated.) The cells were washed once in 10 ml of freshly prepared SED, once in 10 ml of sterile 1M sorbitol, and finally resuspended in 10 ml of SCE buffer. 7.5 µl of 3 mg/ml Zymolyase (100,000 units/g, obtained from Miles Laboratories) was added to the cell suspension. The cells were incubated at 30°C for about 10 minutes. (A reduction of 60% in OD_{600} in 5% SDS can be utilized as a correct time marker.) The spheroplasts were washed in 10 ml of sterile 1 M sorbitol by centrifugation at 700 g for 5-10 minutes. 10 ml of sterile CaS was used as a final cell wash, and the cells were centrifuged again at 700 g for 5-10 minutes and then resuspended in 0.6 ml of CaS.

D. Transformation

Pichia pastoris GS115 cells were transformed with 10 µg of linearized DNA (see step A) using the spheroplast transformation technique of Sreekrishna et al, Gene 59, 115-125 (1987). DNA samples were added (up to 20 µl volume) to 12 x 75 mm sterile polypropylene tubes. (DNA should be in a suitable buffer such as TE buffer or CaS.) 100 µl of spheroplasts were added to each DNA sample and incubated at room temperature for about 20 minutes. 1 ml of PEG solution was added to each sample and incubated at room temperature for about 15 minutes and centrifuged at 700 g for 5-10 minutes. SOS (150 µl) was added to the pellet and incubated for 30 minutes at room temprature. Finally 850 µl of 1M sorbitol was added.

E. Regeneration of Spheroplasts

A bottom agarose layer of 20 ml of regeneration agar SDR was poured per plate at least 30 minutes before transformation samples were ready. In addition, 8 ml aliquots of regeneration agar were distributed to 15 ml conical bottom Corning tubes in a 45°C water bath during the period that transformation samples were in SOS. Aliquots of 50 or 250 µl of the transformed sample was added to the 8 ml aliquots of molten regeneration agar held at 45°C and poured onto plates containing the solid 20 ml bottom agar layer. The plates were incubated at 30°C for 3-5 days.

F. Selection of Transformants

Transformants were selected for by culturing on SDR, a media lacking histidine. The colonies which grew in the absence of histidine were also screened for "methanol-slow" phenotype, indicating displacement of the AOX1 structural gene by the NotI DNA fragment) in the case of transformants obtained using NotI linearized vectors. Several transformed GS115 cells showing "methanol-normal" (those obtained with SstI linearized DNA) and methanol-slow were then cultured and assayed for the production of HSA.

Example V

Methanol Induced Secretion of HSA in *Pichia pastoris* Integrative Transformants

Pichia pastoris GS115 strains transformed with pHSA313, pHSA413, and pPGP1 were analysed for HSA secretion in shake tube cultures. Both methanol-slow and methanol-normal strains were used. In each case 36 independent clones were studied. Transformants obtained with pAO807N served as negative controls. A protocol was developed to ensure efficient secretion and stable accumulation of HSA in the culture medium.

Cells were grown to saturation in 10 ml BMGR or BMGY, and were placed in 50 ml tubes (2-3 days). The cells would be in the range of 10-20 A_{600} units. The cells were harvested, the supernatant liquid was discarded, and then the pellet was resuspended in 2 ml of BMMR or BMMY. The tube was covered with a sterile gauze (cheese cloth) instead of a cap. The tube(s) were then returned to a 30°C shaker. At the end of 2-3 days, the cells were pelleted, and the supernatant assayed for product. The pellets could be resuspended with fresh medium and returned to the shaker for renewed secretion. With *Pichia*-HSA strains, 10 µl of media supernatant was sufficient for analysis by SDS-PAGE followed by Coomassie staining. Under

these conditions a single band of 67 kD corresponding to HSA was observed. There was no significant difference between the expression levels of GS115/pHSA313 vs GS115/pHSA413 transformants, suggesting that deleting the 3' untranslated sequences from the HSA gene present in pHSA313 did not significantly affect expression levels. No significant difference in the HSA expression level was observed between methanol-slow vs methanol-normal transformants, suggesting that disruption of AOX1 was not essential for efficient HSA expression. As expected, HSA was absent in both the culture medium and the cell extract of GS115/pAO807N transformants (negative control). Clonal variants were selected which demonstrated increased levels of HSA secretion.

Example VI

Batch-Fed Fermentation of Mut⁻ *Pichia pastoris* for Production of HSA

Pichia pastoris GS115:pHSA 413-6 and pPGP1-9-6 were inoculated into two 20 liter Biolafitte fermenters with an 8.5 1 working volume. The inoculum was prepared in the following manner: a culture was grown on a YM plate and then transferred to 100 ml YM broth in a shake flask and grown for about 24 hours. 50 mls of this culture was transferred to 1 liter of YM broth in a shake flask and also grown for about 24 hours. 1 liter of this was then transferred to 8.5 liters of fermenter medium in the Biolafitte fermenter. Fermentor medium consisted of Minimal salts + biotin + 5 percent glycerol. Batch growth conditions included the following: pH = 5.8 (controlled with NH_3), temperature = 30° C, and percent dissolved oxygen greater than 20 percent air saturation.

Glycerol exhaustion was complete after about 24 hours, at which time a slow methanol feed was begun at a rate of 10-15 ml/hr. The methanol concentration was monitored in the fermenter and the feed rate was adjusted to maintain a concentration of 0.5-0.9 percent of methanol in the broth.

Secreted HSA in the media was measured quantitatively by densitometry of Coomassie blue stained polyacrylamide gels containing SDS (SDS-PAGE). Areas were referenced to a series of known weights of authentic HSA run on the same SDS-PAGE gels. The data from these gels is included in Tables I and II.

The following Table illustrates the effect of changes in pH on the amount of HSA produced:

Table III

Production of HSA by Batch- Fed Fermentation			
Run	Strain	pH	HSA g/l
1	GS115:pPGP1-9-6	5.09-5.32	0.71
2	GS115:pPGP1-9-6	5.22	0.81
3	GS115:pPGP1-9-6	5.91	1.28
4	GS115:pPGP1-9-6	5.78	1.59
5	GS115:pPGP1-9-6	5.78	1.98
6	GS115:pPGP1-9-6	5.79	1.32

The following Table illustrates the level of HSA production which can be achieved at higher pH levels:

Table IV

Production of HSA by Batch-Fed Fermentation					
Run	Strain	pH	Hours MeOH	Dry Cell Wt.	HSA Broth g/l
1	GS115:pHSA 413-6	5.79	101	ND	2.13
2	GS115:pHSA 413-6	5.85	237	101	3.39
3	GS115:pHSA 413-6	5.85	265	98	2.70
4	GS115:pHSA 413-6	5.97	258	117	2.90
ND = Not Determined					

Example VII

Protocol for Shake Tube and Shake Flask Secretion of Proteins from *P. pastoris*

For efficient secretion and stable accumulation of HSA in shake tubes and shake flasks it is necessary to use a pH of 5.7-6.4 instead of 5.0 or 5.2 for the fermenter media, to add small amounts yeast extract (0.5-0.1%) and peptone (0.1-0.2%) to the fermenter medium and to start inducing expression at a low cell density (20-25 gram dry cell weight/liter). Using these techniques, we have developed a protocol that permits efficient secretion of HSA from cells grown in shake tubes and flasks. We believe that this protocol is applicable in general to secretion of proteins from *Pichia pastoris*.

Shake Tube:

Grow cells to saturation in 10 ml BMGR or BMGY placed in 50 ml tube (2-3 days). The A_{600} of cells will be in the range of 10-20. Harvest cells, discard the supernatant liquid and resuspend the pellet with 2 ml of BMMR or BMMY. Cover the tube with a sterile gauze or cheese cloth instead of the cap. Return the tube(s) to the shaker and maintain the shaker at about 30°C. At the end of 2-3 days, pellet cells, and analyze supernatant for product. The pellet can be resuspended with fresh media and returned to shaker for renewed secretion. With *Pichia*-HSA strains, 10 ul of media supernatant is sufficient for analysis by SDS-PAGE followed by Coomassie staining. Under these conditions, a single band corresponding to HSA size (67 kD) is observed.

Shake Flask:

Grow cells as described above in 1 liter of medium (BMGY or BMGR) in a 2 liters flask. Harvest cells and suspend with 50-75 ml of BMMR or BMMY in a fermenter flask (Tunair™ shake-flask fermentation system, Research Products International Corporation) or a baffled flask covered with cheese cloth. Return to the shaker at 30°C and induce for 2-4 days. At the end of 2-4 days the cells are pelleted and the supernatant is analyzed for product. Shake tubes secretion can be re-initiated by resuspending the pelleted cells in fresh media.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: William D. Prevatt et al.

(ii) TITLE OF INVENTION: Expression of Human Serum Albumin in *Pichia pastoris*

(iii) NUMBER OF SEQUENCES: 3

(IV) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: RICHMOND, PHILLIPS, HITCHCOCK & UMPHLETT

(B) STREET: P.O. Box 2443

(C) CITY: Bartlesville

(D) STATE: OK.

(E) COUNTRY: USA

(F) ZIP: 74005

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM PC

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: Display Write 4

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Hal Brent Woodrow

(B) REGISTRATION NUMBER: 32,501

(C) REFERENCE/DOCKET NUMBER: 32819US

(ix) TELECOMMUNICATION NUMBER: 1-918-661-0624

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 940 bp

(B) TYPE: DNA

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TGCAAACGCA  GGACCTCCAC  TCCTCTTCTC  CTCAACACCC  ACTTTTGCCA  TCGAAAAACC  180
AGCCCAGTTA  TTGGGCTTGA  TTGGAGCTCG  CTCATTCCAA  TTCCTTCTAT  TAGGCTACTA  240
ACACCATGAC  TTTATTAGCC  TGTCTATCCT  GGCCCCCCTG  GCGAGGTTCA  TGTTTGTTTA  300
TTTCCGAATG  CAACAAGCTC  CGCATTACAC  CCGAACATCA  CTCCAGATGA  GGGCTTTCTG  360
AGTGTGGGGT  CAAATAGTTT  CATGTTCCCC  AAATGGCCCA  AAACTGACAG  TTTAAACGCT  420
GTCTTGGAAC  CTAATATGAC  AAAAGCGTGA  TCTCATCCAA  GATGAACTAA  GTTTGGTTCG  480
TTGAAATGCT  AACGGCCAGT  TGGTCAAAAA  GAAACTTCCA  AAAGTCGGCA  TACCGTTTGT  540
CTTGTTTGGT  ATTGATTGAC  GAATGCTCAA  AAATAATCTC  ATTAATGCTT  AGCGCAGTCT  600
CTCTATCGCT  TCTGAACCCC  GGTGCACCTG  TGCCGAAACG  CAAATGGGGA  AACACCCGCT  660
TTTTGGATGA  TTATGCATTG  TCTCCACATT  GTATGCTTCC  AAGATTCTGG  TGGGAATACT  720
GCTGATAGCC  TAACGTTCAT  GATCAAAATT  TAACTGTTCT  AACCCCTACT  TGACAGCAAT  780
ATATAAACAG  AAGGAAGCTG  CCCTGTCTTA  AACCTTTTTT  TTTATCATCA  TTATTAGCTT  840
ACTTTCATAA  TTGCGACTGG  TTCCAATTGA  CAAGCTTTTG  ATTTTAACGA  CTTTTAACGA  900
CAACTTGAGA  AGATCAAAAA  ACAACTAATT  ATTCGAAACG                          940
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(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 600 bp
(B) TYPE: DNA
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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AAAGTAAACC  CCATTCAATG  TTCCGAGATT  TAGTATACTT  GCCCCTATAA  GAAACGAAGG   60
ATTTCAGCTT  CCTTACCCCA  TGAACAGAAA  TCTTCCATTT  ACCCCCCACT  GGAGAGATCC  120
GCCCAAACGA  ACAGATAATA  GAAAAAAGAA  ATTCGGACAA  ATAGAACACT  TTCTCAGCCA  180
ATTAAAGTCA  TTCCATGCAC  TCCCTTTAGC  TGCCGTTCCA  TCCCTTTGTT  GAGCAACACC  240
ATCGTTAGCC  AGTACGAAAG  AGGAAACTTA  ACCGATACCT  TGGAGAAATC  TAAGGCGCGA  300
ATGAGTTTAG  CCTAGATATC  CTTAGTGAAG  GGTGTTCCGA  TACCTTCTCC  ACATTCAGTC  360
ATAGATGGGC  AGCTTTGTTA  TCATGAAGAG  ACGGAAACGG  GCATTAAGGG  TTAACCGCCA  420
AATTATATAA  AAGACAACAT  GTCCCCAGTT  TAAAGTTTTT  CTTTCCTATT  CTTGTATCCT  480
GAGTGACCGT  TGTGTTTAAT  ATAACAAGTT  CGTTTTAACT  TAAGACCAAA  ACCAGTTACA  540
ACAAATTATA  ACCCCTCTAA  ACACTAAAGT  TCACTCTTAT  CAAACTATCA  AACATCAAAA  600
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(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1830 bp
(B) TYPE: DNA
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG	AAG	TGG	GTA	ACC	TTT	ATT	TCC	CTT	CTT	TTT	CTC	TTT	AGC	TCG
Met	Lys	Trp	Val	Thr	Phe	Ile	Ser	Leu	Leu	Phe	Leu	Phe	Ser	Ser
	-35					-30					-25			
GCT	TAT	TCC	AGG	GGT	GTG	TTT	CGT	CGA	GAT	GCA	CAC	AAG	AGT	GAG
Ala	Tyr	Ser	Arg	Gly	Val	Phe	Arg	Arg	Asp	Ala	His	Lys	Ser	Glu
	-20					-15					-10			
GTT	GCT	CAT	CGG	TTT	AAA	GAT	TTG	GGA	GAA	GAA	AAT	TTC	AAA	GCC
Val	Ala	His	Arg	Phe	Lys	Asp	Leu	Gly	Glu	Glu	Asn	Phe	Lys	Ala
	-5					1				5				
TTG	GTG	TTG	ATT	GCC	TTT	GCT	CAG	TAT	CTT	CAG	CAG	TGT	CCA	TTT
Leu	Val	Leu	Ile	Ala	Phe	Ala	Gln	Tyr	Leu	Gln	Gln	Cys	Pro	Phe
10					15					20				
GAA	GAT	CAT	GTA	AAA	TTA	GTG	AAT	GAA	GTA	ACT	GAA	TTT	GCA	AAA
Glu	Asp	His	Val	Lys	Leu	Val	Asn	Glu	Val	Thr	Glu	Phe	Ala	Lys
25					30					35				
ACA	TGT	GTT	GCT	GAT	GAG	TCA	GCT	GAA	AAT	TGT	GAC	AAA	TCA	CTT
Thr	Cys	Val	Ala	Asp	Glu	Ser	Ala	Glu	Asn	Cys	Asp	Lys	Ser	Lue
40					45					50				
CAT	ACC	CTT	TTT	GGA	GAC	AAA	TTA	TGC	ACA	GTT	GCA	ACT	CTT	CGT
His	Thr	Leu	Phe	Gly	Asp	Lys	Leu	Cys	Thr	Val	Ala	Thr	Leu	Arg
55					60					65				
GAA	ACC	TAT	GGT	GAA	ATG	GCT	GAC	TGC	TGT	GCA	AAA	CAA	GAA	CCT
Glu	Thr	Tyr	Gly	Glu	Met	Ala	Asp	Cys	Cys	Ala	Lys	Gln	Glu	Pro
70					75					80				
GAG	AGA	AAT	GAA	TGC	TTC	TTG	CAA	CAC	AAA	GAT	GAC	AAC	CCA	AAC
Glu	Arg	Asn	Glu	Cys	Phe	Leu	Gln	His	Lys	Asp	Asp	Asn	Pro	Asn
85					90					95				
CTC	CCC	CGA	TTG	GTG	AGA	CCA	GAG	GTT	GAT	GTG	ATG	TGC	ACT	GCT
Leu	Pro	Arg	Leu	Val	Arg	Pro	Glu	Val	Asp	Val	Met	Cys	Thr	Ala
100					105					110				
TTT	CAT	GAC	AAT	GAA	GAG	ACA	TTT	TTG	AAA	AAA	TAC	TTA	TAT	GAA
Phe	His	Asp	Asn	Glu	Glu	Thr	Phe	Leu	Lys	Lys	Tyr	Leu	Tyr	Glu
115					120					125				

ATT	GCC	AGA	AGA	CAT	CCT	TAC	TTT	TAT	GCC	CCG	GAA	CTC	CTT	TTC
Ile	Ala	Arg	Arg	His	Pro	Tyr	Phe	Tyr	Ala	Pro	Glu	Leu	Leu	Phe
130					135					140				
TTT	GCT	AAA	AGG	TAT	AAA	GCT	GCT	TTT	ACA	GAA	TGT	TGC	CAA	GCT
Phe	Ala	Lys	Arg	Tyr	Lys	Ala	Ala	Phe	Thr	Glu	Cys	Cys	Gln	Ala
145					150					155				
GCT	GAT	AAA	GCT	GCC	TGC	CTG	TTG	CCA	AAG	CTC	GAT	GAA	CTT	CGG
Ala	Asp	Lys	Ala	Ala	Cys	Leu	Leu	Pro	Lys	Leu	Asp	Glu	Leu	Arg
160					165					170				
GAT	GAA	GGG	AAG	GTT	TCG	TCT	GCC	AAA	CAG	AGA	CTC	AAG	TGT	GCC
Asp	Glu	Gly	Lys	Val	Ser	Ser	Ala	Lys	Gln	Arg	Leu	Lys	Cys	Ala
175					180					185				
AGT	CTC	CAA	AAA	TTT	GGA	GAA	AGA	GCT	TTC	AAA	GCA	TGG	GCA	GTA
Ser	Leu	Gln	Lys	Phe	Gly	Glu	Arg	Ala	Phe	Lys	Ala	Trp	Ala	Val
190					195					200				
GCT	CGC	CTG	AGC	CAG	AGA	TTT	CCC	AAA	GCT	GAG	TTT	GCA	GAA	GTT
Ala	Arg	Leu	Ser	Gln	Arg	Phe	Pro	Lys	Ala	Glu	Phe	Ala	Glu	Val
205					210					215				
TCC	AAG	TTA	GTG	ACA	GAT	CTT	ACC	AAA	GTC	CAC	ACG	GAA	TGC	TGC
Ser	Lys	Leu	Val	Thr	Asp	Leu	Thr	Lys	Val	His	Thr	Glu	Cys	Cys
220					225					230				
CAT	GGA	GAT	CTG	CTT	GAA	TGT	GCT	GAT	GAC	AGG	GCG	GAC	CTT	GCC
His	Gly	Asp	Leu	Leu	Glu	Cys	Ala	Asp	Asp	Arg	Ala	Asp	Leu	Ala
235					240					245				
AAG	TAT	ATC	TGT	GAA	AAT	CAA	GAT	TCG	ATC	TCC	AGT	AAA	CTG	AAG
Lys	Tyr	Ile	Cys	Glu	Asn	Gln	Asp	Ser	Ile	Ser	Ser	Lys	Leu	Lys
250					255					260				
GAA	TGC	TGT	GAA	AAA	CCT	CTG	TTG	GAA	AAA	TCC	CAC	TGC	ATT	GCC
Glu	Cys	Cys	Glu	Lys	Pro	Leu	Leu	Glu	Lys	Ser	His	Cys	Ile	Ala
265					270					275				
GAA	GTG	GAA	AAT	GAT	GAG	ATG	CCT	GCT	GAC	TTG	CCT	TCA	TTA	GCT
Glu	Val	Glu	Asn	Asp	Glu	Met	Pro	Ala	Asp	Leu	Pro	Ser	Leu	Ala
280					285					290				
GCT	GAT	TTT	GTT	GAA	AGT	AAG	GAT	GTT	TGC	AAA	AAC	TAT	GCT	GAG
Ala	Asp	Phe	Val	Glu	Ser	Lys	Asp	Val	Cys	Lys	Asn	Tyr	Ala	Glu
295					300					305				
GCA	AAG	GAT	GTC	TTC	TTG	GGC	ATG	TTT	TTG	TAT	GAA	TAT	GCA	AGA
Ala	Lys	Asp	Val	Phe	Leu	Gly	Met	Phe	Leu	Tyr	Glu	Tyr	Ala	Arg
310					315					320				

AGG CAT CCT GAT TAC TCT GTC GTG CTG CTG CTG AGA CTT GCC AAG
Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys
325 330 335

ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT GCC GCT GCA GAT CCT
Thr Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro
340 345 350

CAT GAA TGC TAT GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT GTG
His Glu Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val
355 360 365

GAA GAG CCT CAG AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG
Glu Glu Pro Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu
370 375 380

CAG CTT GGA GAG TAC AAA TTC CAG AAT GCG CTA TTA GTT CGT TAC
Gln Leu Gly Glu Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr
385 390 395

ACC AAG AAA GTA CCC CAA GTG TCA ACT CCA ACT CTT GTA GAG GTC
Thr Lys Lys Val Pro Gln Val Ser Thr Pro Thr Leu Val Glu Val
400 405 410

TCA AGA AAC CTA GGA AAA GTG GGC AGC AAA TGT TGT AAA CAT CCT
Ser Arg Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His Pro
415 420 425

GAA GCA AAA AGA ATG CCC TGT GCA GAA GAC TAT CTA TCC GTG GTC
Glu Ala Lys Arg Met Pro Cys Ala Glu Asp Tyr Leu Ser Val Val
430 435 440

CTG AAC CAG TTA TGT GTG TTG CAT GAG AAA ACG CCA GTA AGT GAC
Leu Asn Gln Leu Cys Val Leu His Glu Lys Thr Pro Val Ser Asp
445 450 455

AGA GTC ACC AAA TGC TGC ACA GAA TCC TTG GTG AAC AGG CGA CCA
Arg Val Thr Lys Cys Cys Thr Glu Ser Leu Val Asn Arg Arg Pro
460 465 470

TGC TTT TCA GCT CTG GAA GTC GAT GAA ACA TAC GTT CCC AAA GAG
Cys Phe Ser Ala Leu Glu Val Asp Glu Thr Tyr Val Pro Lys Glu
475 480 485

TTT AAT GCT GAA ACA TTC ACC TTC CAT GCA GAT ATA TGC ACA CTT
Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp Ile Cys Thr Leu
490 495 500

TCT GAG AAG GAG AGA CAA ATC AAG AAA CAA ACT GCA CTT GTT GAG
Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala Leu Val Glu
505 510 515

```
CTT GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT
Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu Lys Ala
520                 525                 530

GTT ATG GAT GAT TTC GCA GCT TTT GTA GAG AAG TGC TGC AAG GCT
Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys Ala
535                 540                 545

GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG GGT AAA AAA CTT GTT
Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
550                 555                 560

GCT GCA AGT CAA GCT GCC TTA GGC TTA TAA
Ala Ala Ser Gln Ala Ala Leu Gly Leu  -
565                 570
```

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16bp
(B) TYPE: DNA
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
CGAAACG ATG AAG TGG 16
        Met Lys Trp
```

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19bp
(B) TYPE: DNA
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTACCCACT TCATCGTTT 19

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13bp
(B) TYPE: DNA

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTAGGCTTAT AAG 13

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14bp
(B) TYPE: DNA
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AATTCTTATA AGCC 14

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 231bp
(B) TYPE: DNA
(C) STRANDED: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Linker Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCTTAGGCTT ATAACATCTC TACATTTAAA AGCATCTCAG CCTACCATGA GAATAAGAGA 60
AAGAAAATGA AGATCAAAAG CTTATTCATC TGTGTTTTCT TTTTCGTTGG TGTAAAGCCA 120
ACACCCTGTC TAAAAAACAT AAATTTCTTT AATCATTTTG CCTCTTTTTC TCTGTGCTTC 180
AATTAATAAA AAATGGAAAG AATCTAAAAA AAAAAAAAAA AAAAGGAATT C 231

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20bp
(B) TYPE: DNA
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Oligonucleotide

(ix) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTTAGGCTT ATAAGAATTC 20

Claims

1. An improved process for the secretion of a heterologous protein in transformed *Pichia pastoris* cells comprising:
   (a) cultivating in a fermentation broth transformed *Pichia pastoris* cells capable of expressing a heterologous structural gene encoding a secretion signal sequence and a mature heterologous protein, wherein the signal sequence is operably linked to the sequence encoding the mature heterologous protein, under condition suitable for sustaining the viability of said transformed *Pichia pastoris* cells, under suitable conditions for the expression of said heterologous protein by said *Pichia pastoris* cells, and
   (b) maintaining the pH of said fermentation broth at a pH in the range of 5.7 to 6.4 contemporaneously with the expression of a heterologous protein.

2. The process of claim 1 wherein the transformed *Pichia pastoris* cells are grown in a batch-fed manner during the expression of HSA and/or the pH of the fermentation broth is maintained during expression of the heterologous protein in the range of pH 5.7 to pH 6.0, preferably at a pH of 5.8.

3. The process of claim 2 wherein the fermentation broth contains an effective amount of a suitable minimal salts mixture, growth factors and at least one suitable carbon source selected from methanol, glycerol, sorbitol, glucose, fructose and combinations of two or more thereof to maintain the viability of said transformed *Pichia pastoris* cells.

4. The process of claim 3, wherein after the fermentation broth's carbon source is consumed, the transformed *Pichia pastoris* cells are contacted with methanol wherein the methanol is provided at a rate sufficient to maintain the viability of the *Pichia pastoris* cells in contact therewith and the methanol concentration dose not exceed about 5.0 percent by weight.

5. All improved process for the expression of HSA in transformed *Pichia pastoris* cells comprising:
   (a) cultivating in a fermentation broth transformed *Pichia pastoris* cells capable of expressing HSA under conditions suitably for maintaining the viability of said transformed *Pichia pastoris* cells, under suitable conditions for the expression of HSA by said *Pichia pastoris* cells, and
   (b) contacting said fermentation broth containing the transformed *Pichia pastoris* cells with a suitable amount of added amino acids and peptone to enhance the secretion of HSA contemporaneously with the expression of HSA and,
   (c) optionally, maintaining the pH of the fermentation broth during expression of the heterologous protein in the range of from 5.7 to 6.0.

6. The process of claim 1 or 5 wherein *Pichia pastoris* is transformed with a vector selected from a circular plasmid and a linear plasmid, the latter preferably being a linear integrative site-specific vector.

7. The process of claim 6 wherein said linear integrative site-specific vector contains the following serial arrangement:
(a) a first insertable DNA fragment,
(b) at least one marker gene, and at least one expression cassette containing a heterologous structural gene encoding a signal sequence and a mature heterologous protein, operably linked to a regulatory region and a 3' termination sequence, and
(c) a second insertable DNA fragment
wherein the order of the marker gene and cassette of component (b) may be interchanged, and the first and second insertable DNA fragments employed are homologous with separate portions of the *Pichia pastoris* genome wherein the insertable fragments are in the same relative orientation as exist in the *Pichia pastoris* genome.

8. The process of claim 7, wherein the first insertable DNA fragment and the second insertable DNA fragment are obtained from the DNA sequence of a gene from *Pichia pastoris* selected from the AOX1 gene, the p40 gene, the DAS gene, the GAP gene, the PHO1 gene and the HIS4 gene.

9. The process of claim 7 wherein said expression cassette comprises:
(a) a regulatory region selected from the AOX1 5' regulatory region isolated from *Pichia pastoris*, the p40 5' regulatory region isolated from *Pichia pastoris*, the DAS 5' regulatory region from *Pichia pastoris*, the GAP 5' regulatory region isolated from *Pichia pastoris*, the PHO1 5' regulatory region isolated from *Pichia pastoris*, the acid phosphatase promoter isolated from *Saccharomyces cerevisiae*, the galactosidase promoter isolated from *Saccharomyces cerevisiae*, the alcohol dehydrogenase promoter isolated from *Saccharomyces cerevisiae*, the alpha-mating factor promoter isolated from *Saccharomyces cerevisiae* and the glyceraldehyde 3-phosphate dehydrogenase promoter isolated from *Saccharomyces cerevisiae* operably linked to
(b) a heterologous structural gene encoding a secretion signal sequence and a mature heterologous protein, wherein the mature heterologous protein is selected from the group consisting of tissue plasminogen activator, albumins, lysozyme, interferon and invertase and the secretion signal is selected from the group consisting of a native signal sequence for the heterologous protein, a signal sequence of the *Pichia pastoris* acid phosphatase gene and a signal sequence of the *Saccharomyces cerevisiae* alpha-mating factor gene, and
(c) a 3' termination sequence from *Pichia pastoris* selected from the 3' termination sequence isolated from the AOX1 gene, the p40 gene, the DAS gene, the GAP gene, the PHO1 gene and the HIS4 gene.

10. The process of claim 7, wherein said marker gene is selected from HIS4 isolated from *Pichia pastoris*, ARG4 isolated from *Pichia pastoris*, SUC2 isolated from *Saccharomyces cerevisiae*, G418^R gene of Tn903 and G418^R gene of Tn601.

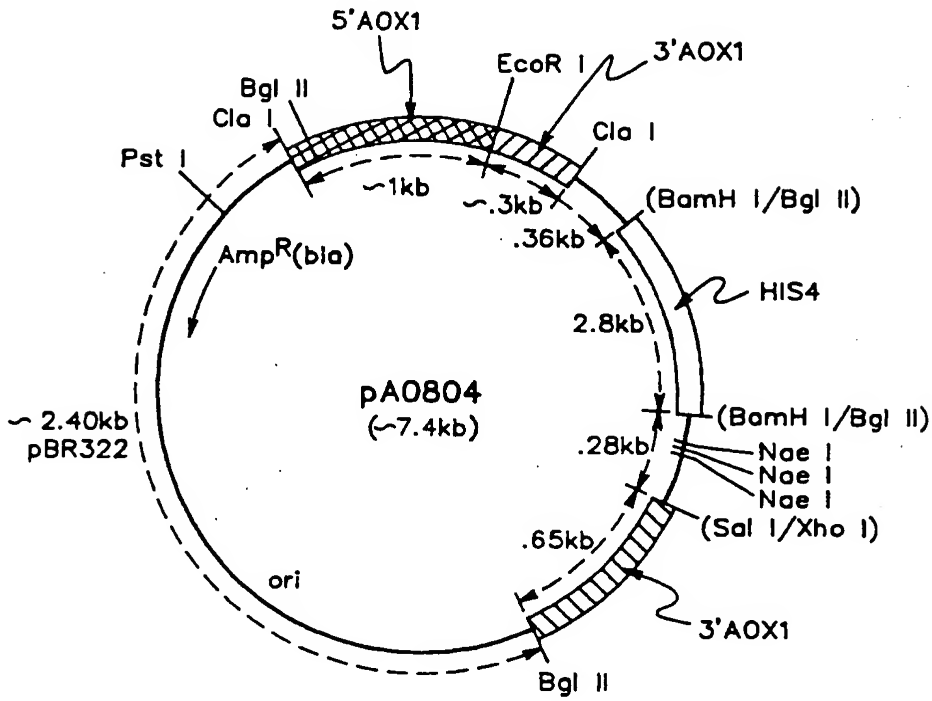
11. The process of claim 9, wherein the heterologous structural gene encodes the HSA native signal sequence operably linked to the sequence encoding the mature HSA protein.

12. The process of claim 9 wherein the plasmid comprises an autonomously replicating DNA sequence and a marker gene, said marker gene being selected from HIS4 isolated from *Pichia pastoris*, ARG4 isolated from *Pichia pastoris*, SUC2 isolated from *Saccharomyces cerevisiae*, G418^R gene of Tn903 and G418^R gene of Tn601.

13. The process of claim 12 wherein said plasmid comprises:
(a) the AOX1 5' regulatory region isolated from *Pichia pastoris* operably linked to
(b) a structural gene for HSA encoding a native signal sequence for HSA and a mature HSA protein, wherein the HSA signal sequence is operably linked to the sequence encoding the mature HSA protein operably linked to
(c) the 3' termination sequence of AOX1 isolated from *Pichia pastoris* operably linked to
(d) at least one marker gene, preferably a HIS4 gene, and
(e) a second DNA fragment which is about a 0.19 kilobase sequence of an autonomously replicating DNA sequence.

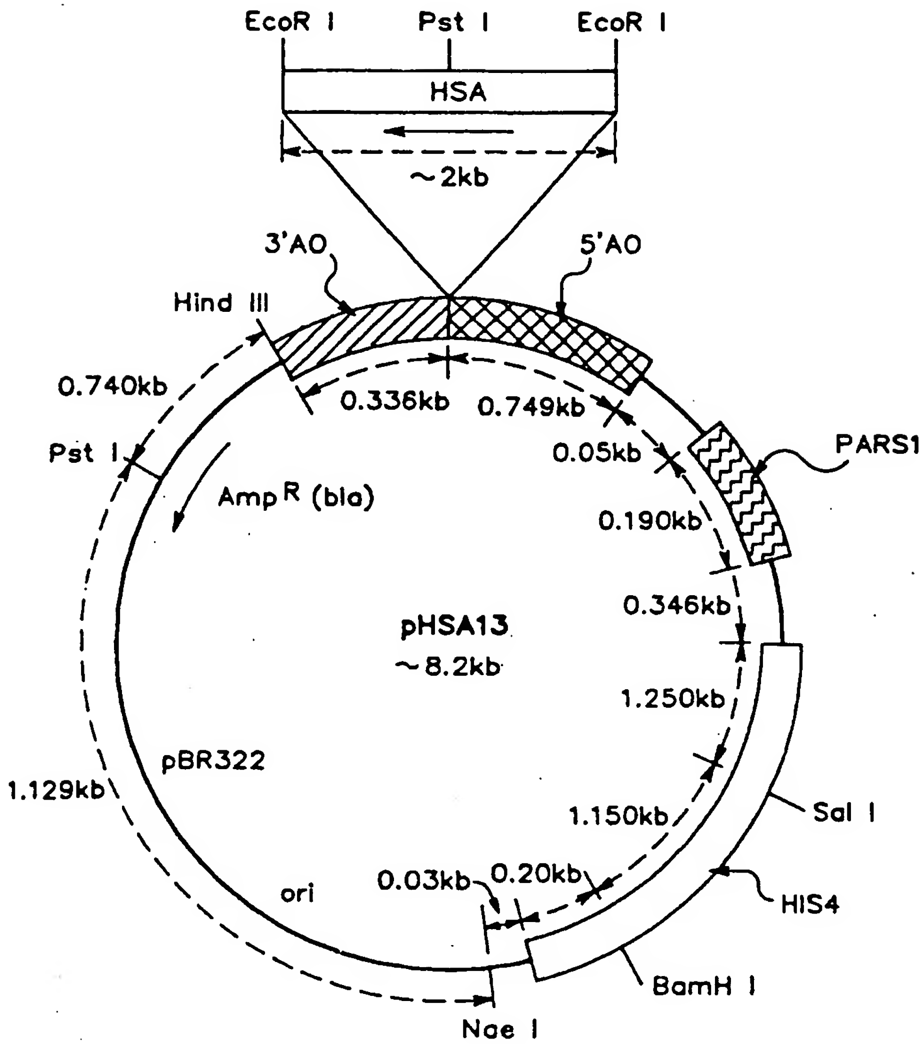
14. The process of claim 5 wherein the amino acids are provided in the form of yeast extract at a

concentration in the range of 1 g/liter to 15 g/liter and/or wherein the peptone is provided at a concentration in the range of 1 g/liter to 50 g/liter.



5'AOX1
EcoR I
3'AOX1
Bgl II
Cla I
Cla I
Pst I
~1kb
~.3kb
(BamH I/Bgl II)
.36kb
AmpR(bla)
HIS4
2.8kb
pA0804
(~7.4kb)
(BamH I/Bgl II)
~2.40kb
pBR322
.28kb
Nae I
Nae I
Nae I
(Sal I/Xho I)
.65kb
ori
3'AOX1
Bgl II

FIG. 1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Pst I
EcoR I
HSA
~2kb
3'AO
5'AO
Hind III
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0.336kb
0.749kb
0.05kb
PARS1
Pst I
Amp R (bla)
0.190kb
0.346kb
pHSA13
~8.2kb
1.250kb
pBR322
1.129kb
1.150kb
Sal I
0.03kb
0.20kb
ori
HIS4
BamH I
Nae I

FIG. 2

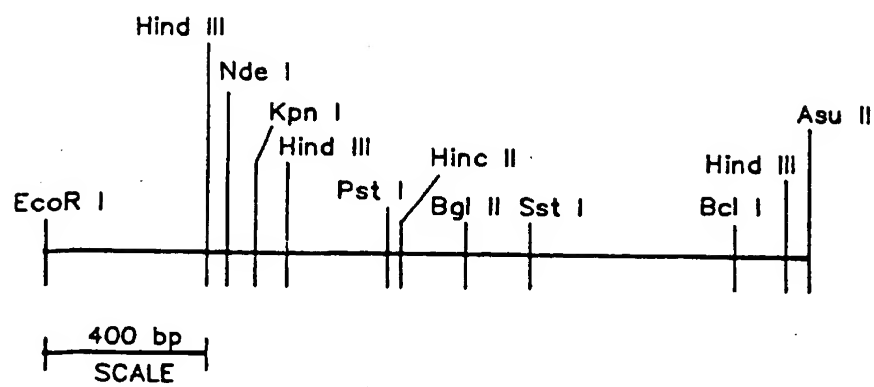
Hind III
Nde I
Kpn I
Hind III
Asu II
Hinc II
Hind III
EcoR I
Pst I
Bgl II
Sst I
Bcl I
400 bp
SCALE

FIG. 3

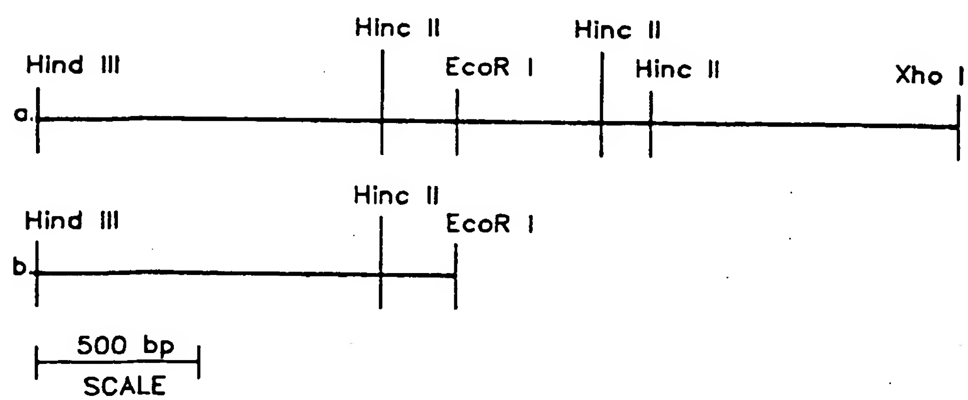
Hinc II
Hinc II
Hind III
EcoR I
Hinc II
Xho I
a.
Hinc II
Hind III
EcoR I
b.
500 bp
SCALE

FIG. 4

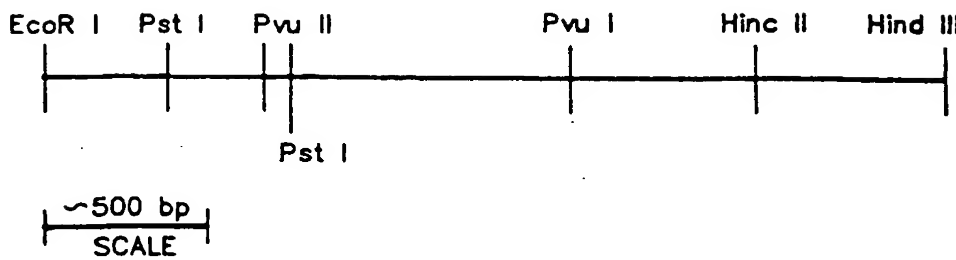
EcoR I
Pst I
Pvu II
Pvu I
Hinc II
Hind III
Pst I
~500 bp
SCALE

FIG. 5

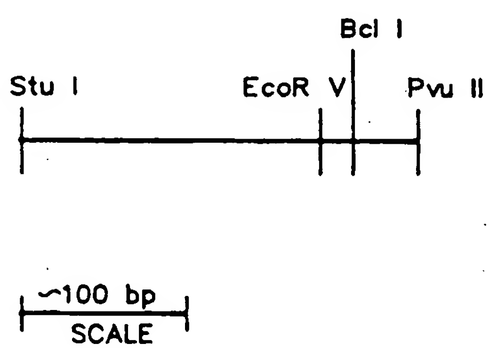
Bcl I
Stu I
EcoR V
Pvu II
~100 bp
SCALE

FIG. 6

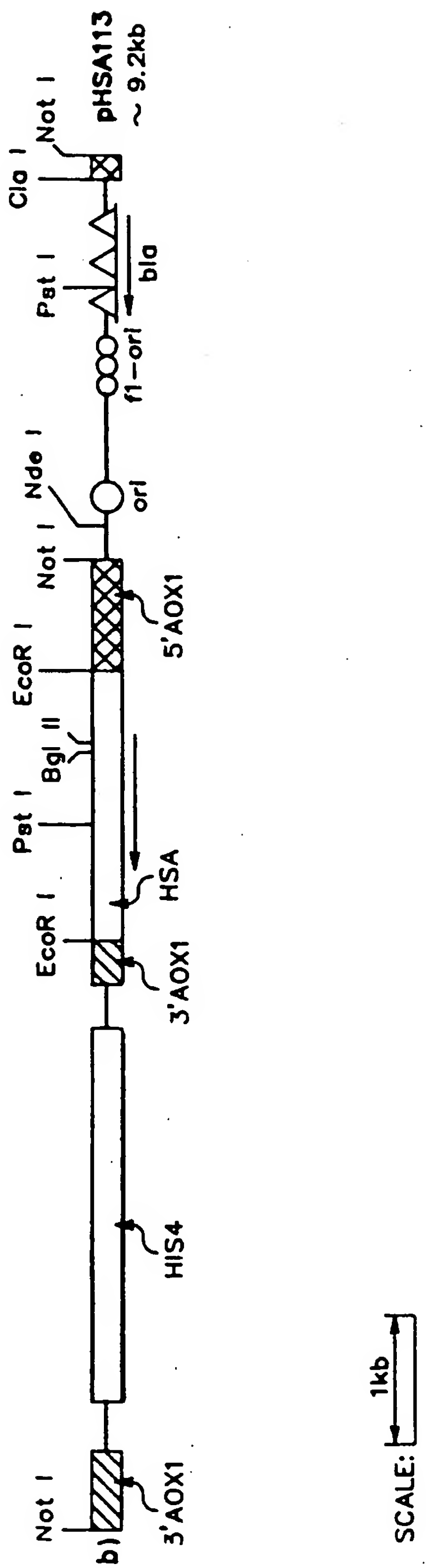
b)
Not I
3'AOX1
HIS4
EcoR I
3'AOX1
Pst I
HSA
Bgl II
EcoR I
Not I
5'AOX1
Nde I
ori
f1-ori
Pst I
bla
Cla I
Not I
pHSA113
~ 9.2kb
SCALE:
1kb

FIG. 7

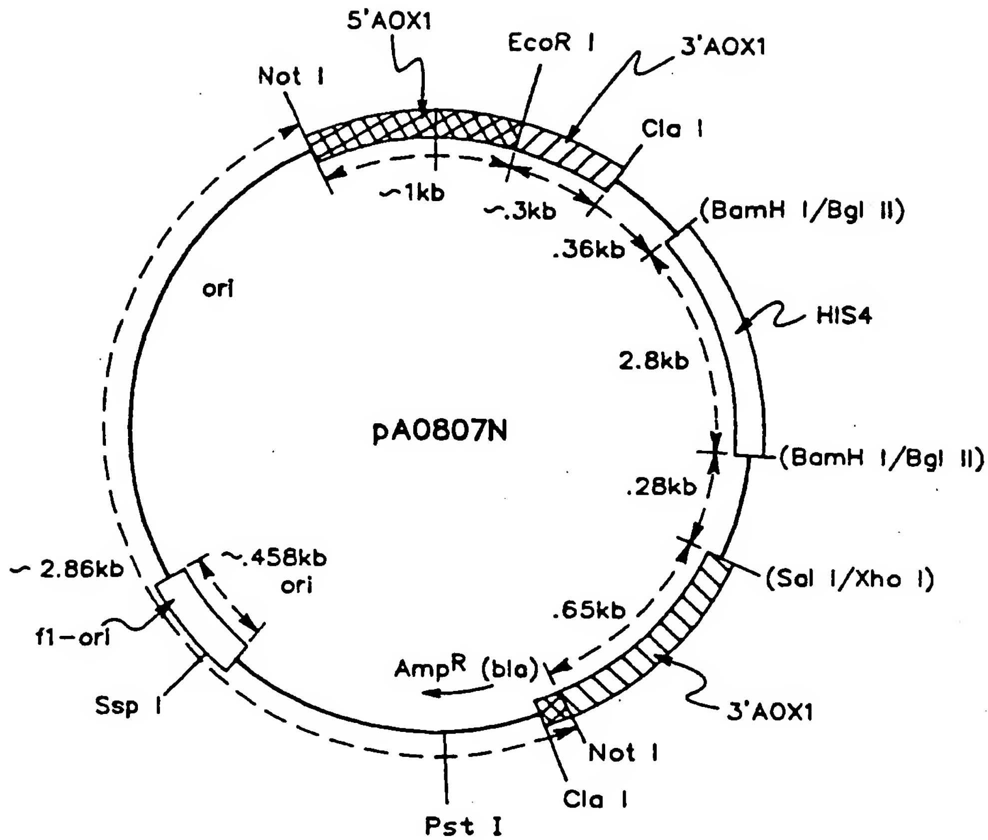
5'AOX1
EcoR I
3'AOX1
Not I
Cla I
~1kb
~.3kb
(BamH I/Bgl II)
.36kb
ori
HIS4
2.8kb
pA0807N
(BamH I/Bgl II)
.28kb
~ 2.86kb
~.458kb
ori
(Sal I/Xho I)
.65kb
f1-ori
AmpR (bla)
Ssp I
3'AOX1
Not I
Cla I
Pst I

FIG. 8